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NOTE

Cloning of a Manganese Peroxidase cDNA Gene Repressed by Manganese in *Trametes versicolor*

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White-rot fungi have the following enzyme systems for lignin degradation: laccase, lignin peroxidase and manganese peroxidase. There are other types of peroxidases related to lignin degradation, one of which we have cloned a cDNA gene of manganese-repressed peroxidase (MrP) in *Trametes versicolor* isolated in South Korea. The *mrp* transcript level has been decreased by 1 μM of Mn^{2+}.

Keywords: lignin degradation, manganese repressed peroxidase, *Trametes versicolor*

After cellulose, lignin is the second most abundant organic carbon material on earth. Since lignin is a notorious, recalcitrant polymer, its degradation is very important in carbon recycling and the removal of wood waste. White-rot fungi can degrade cellulose, hemicellulose and lignin. Therefore, these fungi have been used for the degradations of many other resistant materials. These fungi secrete laccase, lignin peroxidase and manganese-dependent peroxidase, which are strongly related to lignin degradation. *Trametes versicolor*, a white-rot basidiomycete, has long been used for medicinal purposes in Asia including South Korea, and it also has high lignin degrading activity. There are many reports about the lignin degrading enzymes and their related genes in this fungus (Jönsson *et al*., 1995; Johansson and Nymann, 1996; Johansson *et al*., 2002).

Manganese-dependent peroxidases (MnPs) of many white-rot fungi have been studied extensively regarding their biochemical characteristics (Shin, 2004). MnPs oxidize phenolic compounds in the presence of H_2O_2 and manganese. This enzyme oxidizes Mn (II) to Mn (III), and the Mn (III) in turn oxidizes monomeric phenols (Wariishi *et al*., 1988), phenolic lignin dimmers (Wariishi *et al*., 1989), and synthetic lignin (Wariishi *et al*., 1991) via the formation of phenoxy radicals. Along with MnP, lignin peroxidase (LiP) has been reported that this enzyme plays an important role in the degradation of lignin (Schmidt *et al*., 1989). A third type of lignin degrading peroxidase was reported in *Pleurotus eryngii*, which is more similar to LiP than MnP but has Mn (II) binding site (Ruiz-Dueñas *et al*., 1999). This type of peroxidase was designated as a versatile peroxidase by the authors. In the same year, a fourth type of fungal extracellular peroxidase (NPR) for lignin degradation has been reported in *T. versicolor* (Collins *et al*., 1999). This peroxidase has the proposed Mn^{2+}-binding residues of MnP, even though the transcript level is repressed by low concentrations of Mn^{2+}. Therefore, there are at least four kinds of peroxidases in white rot fungi related to lignin and/or recalcitrant material degradation.

Phanerochaete chrysosporium is one of the most widely studied white rot fungi regarding lignin degradation of enzymes and their related genes. However, this fungus has not been reported as a native fungus in South Korea. It is required to study with a white rot fungal strain isolated in South Korea. *T. versicolor* dikaryon has been isolated and degradation of phenanthrene by this fungus was reported (Han *et al*., 2004). Monokaryons (9522-1, 9522-4) from the dikaryon have been generated through protoplast techniques. The genetic transformation to antibiotic resistance was reported (Kim *et al*., 2002). We also got a MnP DNA fragment using the DNA sequences of the metal binding regions for the PCR primers (Kim *et al*., 2003). In the present study, we cloned a peroxidase cDNA gene using the RACE-PCR (rapid amplification of cDNA ends PCR) technique. This showed many similarities with the reported novel extracellular peroxidase by Collins group (1999).

T. versicolor monokaryon was grown at 30°C in YMG medium (Kim *et al*., 2002). The liquid culture was prepared by shaking the cultivation of the liquid medium for

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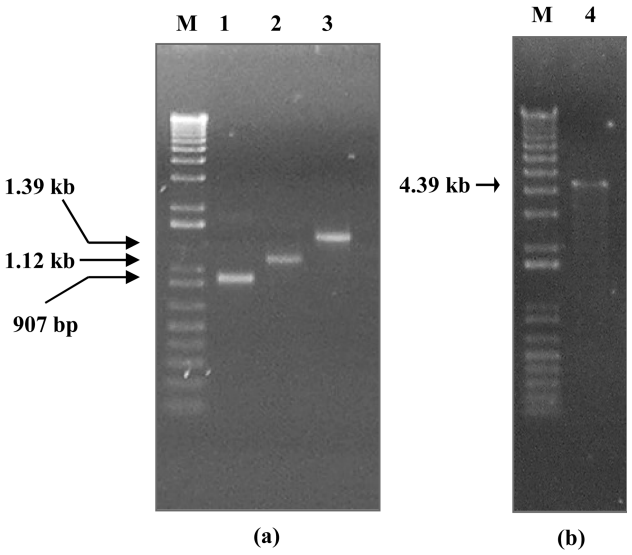

Fig. 1. Agarose gel electrophoresis analysis of the RACE-PCR products. Lanes M, molecular weight markers (1 kb ladders); 1, 5'-RACE-PCR product (907 bp); 2, 3'-RACE-PCR product (1.12 kb); 3, full cDNA product by the second RACE-PCR (1.39 kb); 4, full cDNA cloned into pGEM-T vector cut with *Apa*I. Arrows designate the amplified RACE-PCR products.

four days inoculated with 20 pieces (by cork-borer #1) of mycelial lawn. The whole culture was ground with Waring blender, and then transferred (10% v/v) to a fresh liquid medium.

Total RNAs were isolated from the 36 h culture using the RNeasy Plant Mini Kit (Qiagen, USA) when the peroxidase activity reached its highest level. Full length cDNA was synthesized using a CapFishing cDNA isolation kit (Seegene, South Korea) with 3 μg of total RNA. Based on the sequences of the metal binding regions, we designed degenerated primers (forward primer: 5'-CACGACGCCATCGSCATCTC-3' <F1>, reverse primer: 5'-GTGCGASATSAGCATMCAGAC-3' <R1>), and then extended the 5' and 3'-region using the RACE-PCR technique (Seegene, South Korea). The 3'-region was cloned using the F1 and the 3'-RACE primer, while the 5'-region was amplified using the 5'-RACE primer with another specific reverse primer (R2: 5'-GAGGAAGTCGGACTGGAGAC-3'). We got 907 bp fragment of the 5'-region and 1,120 bp fragment of the 3'-region, and these two 5'- and 3'-flanking regions were assembled to synthesize the full-length cDNA gene (Fig. 1). The primers for the RACE-PCR existed in the full cDNA gene and the predicted ORF consisted of 364 amino acids from the 1,395 bp cDNA (Fig. 2).

When the nucleotide and the predicted amino acid sequences were analyzed through the BLAST program, the cDNA sequence and the deduced amino acid sequence of the ORF showed 96.3% identity (1,054/1,095) and 98.65% similarity, respectively with the unique extracellular peroxidase (NPR) of *T. versicolor* reported earlier

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gatagggaac aaacgacacc cgtcgacgaa ctcgtaggac agcantcggg accgcttcag    60
cacgcttgca ttcgacgacc ttcatcatgt tctccaaggc cctcctctcc atcgtcgctc   120
tcgctgcgtc cttcaccgct gctgtcccgt ccgccaacaa gcgggctacc tgcagcggcg   180
gtcagaccac tgctaacgat gcatgctgcg tgtggttcga cgtcctcgac gacattcaga   240
gcaacctctt ccacggcggc gagtgtggcg agaacgccca cgaatctctc cggctcatct   300
tccacgacgc catcggcatc tcccctgcgt tgacggcggc ggggcagttc ggcggcggag   360
gtgctgacgg ctcgatcatg gcccacaccg acgtcgagat ccagtacgcc gccaacaacg   420
gccttgacga gatcatcgag gagcagcgcc ccttcgcgct caagcacaac gtgtccttcg   480
gcgacttcat ccagttcgcg ggagcggtcg gtgtggcgaa ctgcaacggt ggcccgcaga   540
ttggcttctt cgctggccgc tcgaacgact cgcagcccgc gcccgacaag ctcgtgccgc   600
tccccagcga ctcggtcacc gacatcctcg cacgtgtcgc cgacgcgggc ttcgcccctg   660
tcgagctcgt ctggatgctg atctcgcaca cggtcgccgc gcaggacaag gttgacgact   720
ccatccccgg cacgcccttc gactccaccc cgagcgactt cgacgcccag ttcttcgtcg   780
agtcgatgct caacggcact ctcactcccg gcagcgctct ccacgacggt gaggtccagt   840
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cctcgtgcga gtggcagaag atgatcgccg accgcgcgaa catgctccag aagttcgagc   960
agaccgtgct caagctgtcg ctgctcgggt tctcccagtc ggcgctcact gactgctcgg  1020
acgtcatccc gatcgcgacc ggcacggtcg cggacccctt cctccctgcg ggcaagacga  1080
tggccgacat cgaggctgcg tgcgccgcca cgcccttccc tacgctgtct gccgcgcctg  1140
gtcccgagac caccatcccc gcagtccccc tcgactcgta agatgcggtc atacgatatg  1200
gtggcgggca gtaatgtctg gcacgatgaa cggacttgat tggaagtaga taactttatg  1260
atccttggta ttacggtgga acttgaaggg cttcgcgtac gagctgatta ggtctatttg  1320
ttgaacgtga tgtgatacgc tatcgtcttc aaaatgcatt ccttgctgct ccttaaaaaa  1380
aaaaaaaaaa aaaaa  1395
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Fig. 2. Nucleotide sequence of manganese-repressed peroxidase cDNA gene (1,395 bases). Start and stop codons are boxed. F1, R1 and R2 primers are shown as the double, single wavy, and dotted line respectively. Sequences with single underline represent the primers used for the confirmation of *mrp* by RT-PCR.

(Fig. 3) (Collins *et al.*, 1999; GenBank accession No. AF008585). Given this result, this cDNA was designated as an additional manganese repressed peroxidase (MrP) of *T. versicolor* (EMBL Data Library accession No. AJ745080).

In order to confirm that this gene was repressed by extracellular manganese, $MnSO_4$ (final concentration; 1.0 μM) was added to the YMG culture medium. The fungus was subsequently grown for three days under the same conditions as earlier. The total RNAs were isolated and the *mrp* expression was examined by the RT-PCR with the *mrp*-specific primers (forward primer: 5'-TCATGGCCCACACCGACG-3', reverse primer: 5'-GCGGTCGGCGATCATCTT-3'), and the expected amplified fragment was 560 bp.

As in Fig. 4, the transcript level of the cloned gene in *T. versicolor* was repressed by the low concentration of Mn^{2+} (1 μM). These data suggest that *T. versicolor* isolated in South Korea has another manganese-repressed peroxidase (*mrp*) which indicates a high degree of similarity with the unique extracellular peroxidase gene (npr) reported by Collins group (Collins *et al.*, 1999). In that study, expression was also inhibited by the low concen-

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TvMRP   MFSKALLSIVALAASFTAAVPSANKRATCSGGQTTANDACCVWFDVLDDI
TvNPR   MFSKALLSIVALAASFTAAVPSANKRATCSGGQTTANDACCVWFDVLDDI

TvMRP   QSNLFHGGECGENAHESLRLIFHDAIGISPALTAAGQFGGGGADGSIMAH
TvNPR   QSNLFHGGECGENAHESLRLIFHDAIAFSPALTAAGQFGGGGADGSIMAH

TvMRP   TDVEIQYAANNGLDEIIEEQRPFALKHNVSFGDFIQFAGAVGVANCNGGP
TvNPR   TDVEIQYAANNGLDEIIEEQRPFALKHNVSFGDFIQFAGAVGVANCNGGP

TvMRP   QIGFFAGRSNDSQPAPDKLVPLPSDSVTDILARVADAGFAPVELVWMLIS
TvNPR   QIGFFAGRSN DSQPAPDKLVPLPSDSVTDILARVADAGFAPVELVWMLIS

TvMRP   HTVAAQDKVDDSIPGTPFDSTPSDFDAQFFVESMLNGTLTPGSALHDGEV
TvNPR   HTVAAQDKVDDSIPGTPFDSTPSDFDAQFFVESMLNGTLTPGSALHDGEV

TvMRP   QSPLPGEFRLQSDFLIGRDSRTSCEWQKMIADRANMLQKFEQTVLKLSLL
TvNPR   QSPLPGEFRLQSDFLIGRDSRTSCEWQKMIADRANMLQKFEQTVLKL--L

TvMRP   GFSQSALTDCSDVIPIATGTVADPFLPAGKTMADIEAACAATPFPTLSAA
TvNPR   GFSQSALTDCSDVIPIATGTVADPFLPAGKTMADIEAACAATPFPTLSAA

TvMRP   PGPETTIPAVPLDS*
TvNPR   SGPETTIPAVPLDS*
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Fig. 3. Comparison of amino acid sequence of MrP with the reported Npr (Collins *et al*., 1999). Different amino acids are boxed in MrP. *designates stop codon.

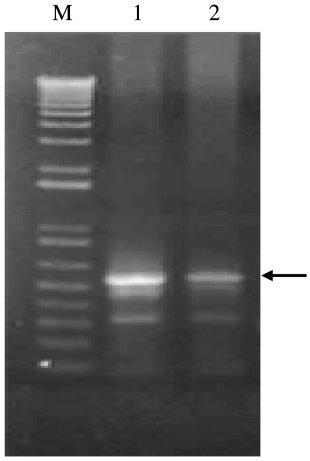

Fig. 4. Agarose gel electrophoresis analysis of the *mrp*-specific RT-PCR product. Lanes M, molecular weight markers; 1, *mrp*-specific RT-PCR product in the control YMG culture; 2, *mrp*-specific RT-PCR product from YMG with $MnCl_2$ (1 μM) culture. Arrow designates the *mrp*-specific RT-PCR product (560 bp).

tration levels of Mn^{2+} (0.5 μM). It looks unlikely that an enzyme has the Mn^{2+} binding domains, but its transcription is inhibited by manganese. Even though the role of MrP is still not clear, a possible role by the enzyme is responsible for later stages of lignin degradation when all Mn^{2+} is oxidized to MnO_2 (Collins *et al*., 1999). As the enzyme systems for lignin degradation work together for the successful degradation, and as the enzyme systems are related in the degradations of various recalcitrant materials, the regulation of MrP activity must also be dependent on the circumstances of the fungus.

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